Biochem. J. (1978) **174**, 989–998
Printed in Great Britain

Maximum Activities and Effects of Fructose Bisphosphate on Pyruvate Kinase from Muscles of Vertebrates and Invertebrates in Relation to the Control of Glycolysis

By VICTOR A. ZAMMIT,* ISIDORUS BEIS† and ERIC A. NEWSHOLME†
**Laboratory of the Marine Biological Association, Citadel Hill, Plymouth PL1 2PB, U.K., and*
†Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

(*Received* 26 *April* 1978)

1. Comparison of the maximum activities of pyruvate kinase with those of phosphofructokinase in a large number of muscles from invertebrates and vertebrates indicates that, in general, in any individual muscle, the activity of pyruvate kinase is only severalfold higher than that of phosphofructokinase. This is consistent with the suggestion, based on mass-action ratio data, that the pyruvate kinase reaction is non-equilibrium in muscle. However, the range of activities of pyruvate kinase in these muscles is considerably larger than that of phosphofructokinase. This difference almost disappears if the enzyme activities from muscles that are known to possess an anaerobic 'succinate pathway' are excluded. It is suggested that, in these muscles, phosphofructokinase provides glycolytic residues for both pyruvate kinase (i.e. glycolysis) and phosphoenolpyruvate carboxykinase (i.e. the succinate pathway). This is supported by a negative correlation between the activity ratio, pyruvate kinase/phosphofructokinase, and the activities of nucleoside diphosphokinase in these muscles, since high activities of nucleoside diphosphokinase are considered to indicate the presence of the succinate pathway. 2. The effect of fructose bisphosphate on the activities of pyruvate kinase from many different muscles was studied. The stimulatory effect of fructose bisphosphate appears to be lost whenever an efficient system for supply of oxygen to the muscles is developed (e.g. insects, squids, birds and mammals). This suggests that activation of pyruvate kinase is important in the co-ordinated regulation of glycolysis in anaerobic or hypoxic conditions, when the change in glycolytic flux during the transition from rest to activity needs to be large in order to provide sufficient energy for the contractile activity. However, lack of this effect in the anaerobic muscles of the birds and mammals suggests that another metabolic control may exist for avian and mammalian pyruvate kinase in these muscles.

A comparison of mass-action ratios with the equilibrium constant for the pyruvate kinase (EC 2.7.1.40) reaction from a large number of muscles from many different animals (Beis & Newsholme, 1975) indicates that the reaction is non-equilibrium in all muscles studied. If the pyruvate kinase reaction is non-equilibrium, the activity of the enzyme must be regulated by changes in the concentrations of phosphoenolpyruvate, ADP or allosteric effectors, but it cannot be regulated by changes in the concentration of its products via mass-action effects. However, preliminary studies indicated that, in some muscles, the activities of pyruvate kinase were considerably higher than those of phosphofructokinase (EC 2.7.1.11) or phosphorylase, which suggested that pyruvate kinase catalysed a near-equilibrium reaction. In this case, the activity must be regulated only by mass-action effects of substrates and products (see Crabtree & Newsholme, 1975; Crabtree, 1976). To provide further information on the relative activities of pyruvate kinase and phosphofructokinase in muscles, maximum activities of the two enzymes from a large number of muscles from different animals have been studied. In addition, a comparative study has been carried out on the activatory effect of fructose bisphosphate on the activity of pyruvate kinase. It has been suggested previously that this effect of fructose bisphosphate is restricted to the enzyme from the muscles of poikilotherms (Mustafa & Hochachka, 1971), but preliminary studies in our laboratory showed that this was not the case. The results of a more detailed comparative study of this effect are presented in this paper.

Materials and Methods

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., except for the following: EDTA (disodium

salt) and all inorganic reagents were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.

Source of animals

Animals were obtained from the sources given by Newsholme & Taylor (1969), Beis & Newsholme (1975) and Zammit & Newsholme (1976), except for the pig roundworm, which was obtained from a local slaughterhouse. Locusts were used 7–14 days after the final moult. Flies were used 7–14 days after emerging from pupae. All other insects were of undetermined age, but they were known to be capable of flight. Apart from rats and mice, for which only male animals were used, muscle tissue was obtained from male and female animals indiscriminately.

Preparation of homogenates

Animals were killed and the muscles dissected rapidly. Muscle tissue was homogenized in ground-glass homogenizers with 10–50 vol. of extraction medium at 0°C. The extraction medium for pyruvate kinase contained 50mM-triethanolamine hydrochloride, 1mM-EDTA, 2mM-$MgCl_2$ and 30mM-2-mercaptoethanol and was adjusted to pH7.5 with KOH. When the effect of fructose bisphosphate was to be studied, the homogenates were either dialysed for a period of 4h or fractionated with $(NH_4)_2SO_4$ (40–60% saturation; approx. 95% of the total activity was precipitated) and dialysed against excess extraction buffer for 2–4h. The extraction medium for phosphofructokinase contained 50mM-Tris/HCl, 1mM-EDTA and 5mM-$MgSO_4$ at pH8.2 (Opie & Newsholme, 1967).

Assay of enzyme activities

Both enzymes were assayed by following the change in A_{340} in a Gilford recording spectrophotometer (model 240) at 25°C. Pyruvate kinase was assayed by a modification of the method of Bücher & Pfleiderer (1955). The assay medium for measurement of maximal activities contained 160mM-triethanolamine hydrochloride, 10mM-$MgCl_2$, 80mM-KCl, 0.17mM-NADH, 5mM-ADP, 2mM-phosphoenolpyruvate and 25μg of lactate dehydrogenase to which 5–10μl of homogenate was added. (Preliminary experiments established that, at this concentration of phosphoenolpyruvate, maximal activities of pyruvate kinase were obtained and that addition of fructose bisphosphate had no further effect.) The final volume in the cuvette was 2.0ml and the final pH was 7.35. The assay was initiated by addition of phosphoenolpyruvate. Controls from which phosphoenolpyruvate was omitted were run concurrently. When the kinetic properties of pyruvate kinase were studied, the concentrations of some of the components were different from above (see Tables for details) and fructose bisphosphate was added as indicated in the legends to Figures and Tables. The effects of fructose bisphosphate on pyruvate kinase were carried out at the pH optimum of the enzyme from each animal. Since fructose bisphosphate shifts the pH optimum of the oyster enzyme from 8.2 to 7.1, its effects can be of a dual nature, and interpretation of the dependence of activities on concentration of fructose bisphosphate must be made with caution. Phosphofructokinase was assayed as described by Zammit & Newsholme (1976), except that 5μg of antimycin A (per cuvette) replaced 1mM-KCN.

Results and Discussion

Control experiments on conditions of pyruvate kinase assay

One aim of this study is to provide information on the maximal activities of pyruvate kinase from a variety of muscles. In such an analysis, the possibility of variation in the properties of the enzyme from one animal to another poses a particular problem. For example, some of the results given below demonstrate that the effect of fructose bisphosphate on pyruvate kinase varies from one animal to another (see below). In the present work, the effects of ions, substrate and ATP concentrations were investigated on the enzyme from muscles of selected animals representing the major phyla investigated (see below). It was established that 75mM-K^+ activated the enzyme optimally. At lower concentrations of K^+, low concentrations (e.g. 1mM) of NH_4^+ activated the enzyme, but there was no effect at high concentrations of K^+. The optimal concentration of Mg^{2+} was 6–10mM; higher concentrations inhibited the enzyme. The K_m values for phosphoenolpyruvate were 0.10, 0.20, 0.12, 0.10 and <0.06mM for the enzyme from locust flight, lobster abdominal, domestic-fowl pectoral, pheasant pectoral and frog gastrocnemius muscles respectively. These values were obtained at saturating concentrations of the other substrate (and cofactors). The concentrations of ATP that produced half-maximal inhibition of the enzyme were 10mM for locust flight muscle and frog gastrocnemius muscle (at 1mM-phosphoenolpyruvate and 5mM-ADP) and 4.6mM for pheasant pectoral and rat heart (at 0.1mM-phosphoenolpyruvate and 2.1mM-ADP). Since the muscle is diluted approx. 1000-fold when the extract is added to the cuvette, it is very unlikely that the ATP concentration in the cuvette would cause inhibiton of pyruvate kinase.

In general, the pH optima for pyruvate kinase (e.g. from frog sartorius, leg muscles of the horse-

shoe crab and abdominal muscles of the lobster) were approx. 7.4. Differences in pH optima for the enzyme from other muscles, which may be important in metabolic regulation, are discussed in detail by Zammit & Newsholme (1978). The activation of the pyruvate kinase from different muscles by fructose bisphosphate is discussed in detail below.

To obtain information about the kinetics of the enzymes that might be applicable *in vivo*, purification of muscle homogenates was kept to a minimum. Thus either a crude extract or an extract obtained after $(NH_4)_2SO_4$ precipitation were used to study the properties. Consequently, the extracts contain other enzymes which could interfere in the assay or modify the concentration of effector molecules added to the cuvette (e.g. aldolase could decrease the concentration of fructose bisphosphate by formation of triose phosphates). In all instances, control assays, in which the substrates of the enzymes under study were omitted, were run concurrently. In these controls the rate of change in A_{340} was never greater than 20% of that due to enzyme activity. In preliminary experiments with some crude extracts, the concentration of fructose bisphosphate present in the cuvette after 3–5min incubation was measured by the usual procedures; more than 80% of the bisphosphate was recovered.

Maximum activities of pyruvate kinase and phosphofructokinase

In all muscles investigated, except those of pig roundworm, oyster and flight muscles of the waterbug, the activity of pyruvate kinase in any individual muscle is only severalfold higher than twice that of phosphofructokinase (Table 1). However, this difference is an order of magnitude in the red muscle of the trout. Except for the muscles mentioned above, the mean ratio, activity of pyruvate kinase/twice the activity of phosphofructokinase, was 3.6. If both enzymes catalyse non-equilibrium reactions and approach saturation with respect to their substrates, then similar maximum activities *in vitro* might be expected [as has been found for phosphorylase and phosphofructokinase (Crabtree & Newsholme, 1972, 1975; Zammit & Newsholme, 1976)]. However, it is likely that the concentrations *in vivo* of the substrates for pyruvate kinase are below the K_m values [K_m values for phosphoenolpyruvate are reported above and concentrations of phosphoenolpyruvate in muscle have been measured by Beis & Newsholme (1975)], so that the activities of this enzyme *in vivo* will be lower than the maximum activities measured *in vitro*. It is concluded that pyruvate kinase catalyses a non-equilibrium reaction in muscle tissue, so that its activity can be controlled by allosteric effectors in addition to changes in the concentrations of its substrates, ADP and phosphoenolpyruvate.

The range of the maximum activities of pyruvate kinase in the muscles studied is remarkably large (almost 7000-fold, i.e. 0.29–2017 μmol/min per g of fresh muscle, values for the body wall of pig roundworm and pectoral muscle of the pheasant respectively), whereas that for phosphofructokinase is considerably less (232-fold, i.e. 0.8–186 μmol/min per g of fresh muscle, values for the catch adductor of oyster and pectoral muscle of pheasant respectively; see Table 1). Maximum activities of phosphofructokinase can provide an indication of the maximum flux through glycolysis (Crabtree & Newsholme, 1972, 1975; Zammit & Newsholme, 1976). Since pyruvate kinase catalyses a non-equilibrium reaction (see above) and is a constituent reaction of glycolysis, it must respond to the glycolytic flux transmitted by phosphofructokinase. Therefore it would be expected that the ranges of activities of the two enzymes should be similar [as has been shown for phosphorylase and phosphofructokinase in muscle (Crabtree & Newsholme, 1972, 1975; Zammit & Newsholme, 1976)]. The difference in ranges suggests that some glycolytic residues may be metabolized by a process not involving pyruvate kinase. This process could be the conversion of phosphoenolpyruvate into succinate (or other end products, e.g. propionate) via the phosphoenolpyruvate carboxykinase reaction (Awapara & Simpson, 1967; Saz, 1971). It is therefore of interest that, if the pyruvate kinase activities of the muscles thought to be involved in the 'succinate pathway' [i.e. pig roundworm body wall and muscles of some marine invertebrates (Zammit & Newsholme, 1976)] are excluded from consideration, the range of pyruvate kinase activities is only 142-fold (lantern retractor muscle of sea urchin to pheasant pectoral muscle), which is similar to that for phosphofructokinase. This suggests that, in some muscles, phosphofructokinase may provide glycolytic residues for only the normal glycolytic process, whereas in other muscles it may provide residues for glycolysis plus the 'succinate pathway' (e.g. roundworm muscle, phasic adductor muscles of oyster, sand gaper and mussel). Zammit & Newsholme (1976) have suggested that the activity of nucleoside diphosphokinase provides a qualitative indication of the importance of the succinate pathway in any given muscle. The latter cannot be indicated by the activities of the enzyme, phosphoenolpyruvate carboxykinase, since this enzyme is present in muscles in which the succinate pathway is considered to be unimportant [e.g. lobster abdominal muscle (Zammit & Newsholme, 1978) and some vertebrate muscles (Crabtree *et al.*, 1972)]. If a low pyruvate kinase activity/2× phosphofructokinase activity ratio is indicative of the presence of the succinate pathway in muscle (see above), there should be a negative correlation between the magnitude of this ratio and the activity of nucleoside diphosphokinase in muscles in which the succinate

Table 1. *Maximal activities of pyruvate kinase and phosphofructokinase in muscle of invertebrates and vertebrates*

Enzyme activities are presented as means, with the ranges and the numbers of separate animals used in parentheses. Some mean activities of phosphofructokinase are taken from Zammit & Newsholme (1976) (ranges and numbers of animals not given).

Animal	Muscle	Enzyme activities (μmol/min per g fresh wt. at 25°C)		Pyruvate kinase activity/ 2 × phosphofructokinase activity ratio
		Pyruvate kinase	Phosphofructokinase	
Coelenterata (Anthozoa)	Basilar	7.6 (7.1–7.8) (3)	1.2	3.2
Sea anemone (*Metridium senile*)	Sphincter	8.6 (8.3, 8.8) (2)	0.9	4.8
Aschelminthes				
Pig roundworm (*Ascaris lumbricoides*)	Body wall	0.3 (0.2–0.3) (10)	7.9 (7.5–8.3) (10)	0.02
Annelida (Polychaeta)				
Sea mouse (*Aphrodite aculeata*)	Longitudinal	44.8 (43.4–45.8) (3)	8.6	2.6
Ragworm (*Nereis virens*)	Dorsal longitudinal	76.0 (73.8–80.0) (3)	10.3	3.7
Mollusca (Bivalvia)	Phasic adductor	54.4 (43.2–77.8) (5)	9.4	2.9
Great scallop (*Pecten maximus*)	Catch adductor	21.0 (16.8–25.7) (3)	4.5	2.3
Variegated scallop (*Chlamys varius*)	Phasic adductor	28.9 (20.3–53.1) (4)	3.2	4.5
	Catch adductor	22.0 (19.5–27.7) (4)	3.8	2.9
Horse mussel (*Modiolus modiolus*)	Phasic adductor	8.8 (7.0–10.1) (3)	4.4	1.0
	Catch adductor	9.1 (6.1–10.0) (3)	2.9	1.6
Striped venus (*Venus striatula*)	Phasic adductor	27.0 (18.0–35.0) (3)	7.3	1.8
Common oyster (*Ostrea edulis*)	Phasic adductor	4.2 (3.3–5.1) (4)	2.8	0.7
	Catch adductor	1.1 (1.0, 1.1) (2)	0.8	0.7
Sand gaper (*Mya arenaria*)	Adductor	6.7 (5.1–7.6) (3)	2.6	1.3
Razor clam (*Ensis ensis*)	Pedal retractor	48.6 (40.8–56.3) (3)	6.5	3.7
Gastropoda				
Common top shell (*Mondonta turbinata*)	Pedal retractor	32.0 (25.6–34.7) (3)	5.2	3.1
Periwinkle (*Littorina*) *littorea*	Pedal retractor	61.4 (41.0–94.0) (5)	12.4	2.5
Thick top shell (*Monodonta lineata*)	Pedal retractor	28.2 (24.9–31.5) (3)	10.9	1.3
Common whelk (*Buccinum undatum*)	Radular retractor	140 (126–154)(3)	19.6	3.6
Common limpet (*Patella vulgata*)	Radular retractor	67.2 (66.5, 67.9) (2)	13.3	2.5
Winkle (*Murex trunculus*)	Radular retractor	71.5 (71.1, 72.0) (2)	23.8	1.5
Arthropoda (Crustaceae)	Deep abdominal flexor	149 (102–192) (4)	9.9	7.9
Lobster (*Homarus vulgaris*)	Claw adductor	59.8 (43.3–74.0) (4)	6.0	5.0

Squat lobster (*Galathea squamifera*)	Abdominal flexor	85.6 (78.1, 93.0) (2)	6.5	6.6
Mediterranean crab (*Pachygraprus marmoratus*)	Leg	55.8 (52.5, 59.0) (2)	9.0	3.1
	Claw adductor	39.6 (36.9–42.2) (3)	12.7	1.6
Swimming crab (*Portunus puber*)	Leg	46.4 (40.6–57.7) (4)	15.0	1.5
Edible crab (*Cancer pagarus*)	Claw adductor	56.4 (47.8–71.3) (3)	9.6	2.9
Pedunculate barnacle (*Lepas anatifera*)	Closer	23.9 (21.4, 26.4) (2)	5.4	2.2
Chelicerata				
Horse-shoe crab (*Limulus polyphemus*)	Leg	153 (121–175) (3)	13.8	5.5
Insect				
Locust (*Schistocerca gregaria*)	Flight	186 (166–213) (4)	21.6 (19.3–22.5) (4)	4.3
Cockroach (*Periplaneta americana*)	Flight	277 (242–307) (3)	21.0 (19.0–22.3) (3)	6.6
Cockroach (*Blaberus discoidalis*)	Flight	89.6 (72.0–100) (3)	11.0 (10.5–12.5) (3)	4.1
Waterbug (*Lethocerus cordofanus*)	Flight	17.9 (12.3–23.4) (4)	12.1 (9.9–14.8) (3)	0.7
Cockchafer (*Melolontha melolontha*)	Flight	131 (99–166) (3)	18.0 (14.8–21.2) (3)	3.6
Rosechafer (*Pachnoda ephippiata*)	Flight	231 (208–248) (3)	32.4 (23.8–36.8) (3)	3.6
Bumble bee (*Bombus* sp.)	Flight	330 (248–378) (6)	21.1 (20.3–22.0) (2)	7.8
Blowfly (*Calliphora vicinia*)	Flight	448 (403–526) (4)	49.2 (47.5–51.7) (4)	4.5
Echinodermata (Echinoidea)				
Common sea-urchin (*Echinus esculentus*)	Lantern retractor	14.2 (11.0–18.1) (4)	1.4	5.1
Pisces				
Dogfish (*Scylliorhinus canicula*)	Heart	103 (87–127) (3)	17.3 (13.4–21.9) (3)	3.0
	Red	101 (60–132) (3)	12.1 (10.9–13.7) (3)	4.2
	White	272 (196–322) (3)	30.1 (22.6–40.8) (3)	4.5
Trout (*Salmo gairdneri*)	Heart	233 (168–343) (4)	43.1 (40.3–46.0) (4)	2.7
	Red	310 (299, 311) (2)	13.9 (13.4, 14.4) (2)	11.1
	White	1225 (1126, 1324) (2)	101 (80.8, 121) (2)	6.1
Goldfish (*Carassius auratus*)	White	34.7 (30.0–44.1) (3)	4.3 (3.9–4.7) (3)	4.0
Amphibia				
Frog (*Rana temporaria*)	Heart	136 (91–169) (5)	20.0 (16.5–21.7) (5)	3.4
	Gastrocnemius	407 (289–613) (6)	37.0 (28.5–52.5) (6)	5.5
Aves				
Domestic pigeon (*Columba livia*)	Heart	125 (106–146) (3)	17.3 (15.6–20.4) (3)	3.6
	Pectoral	334 (259–379) (3)	24.0*	4.8

Table 1.—*continued*

Animal	Muscle	Enzyme activity (μmol/min per g fresh wt. at 25°C)		Pyruvate kinase activity/ 2 × phosphofructokinase activity ratio
		Pyruvate kinase	Phosphofructokinase	
Aves—continued				
Mallard (*Anas platyrhynchos*)	Heart	113 (100–124) (3)	18.9 (14.4–22.1) (3)	3.0
	Pectoral	700 (642–763) (3)	71.9 (50.1–88.7) (3)	4.9
Domestic fowl (*Gallus gallus*)	Heart	93.5 (81–107) (4)	17.6 (14.0–24.6) (4)	2.6
	Pectoral	1508 (1155–1697) (4)	144 (113–194) (4)	5.2
Pheasant (*Phasianus colchicas*)	Heart	126 (118–136) (4)	17.1 (13.5–19.8) (4)	3.7
	Pectoral	2017 (1611–2716) (4)	186 (168–215) (4)	5.4
Mammalia				
Laboratory rat	Heart	144 (118–167) (4)	14.4 (10.8–17.9) (4)	5.0
	Gastrocnemius	866 (766–944) (4)	66.4 (50.7–78.8) (4)	6.5
Laboratory mouse	Heart	188 (150–225) (4)	22.9 (20.5–25.9) (4)	4.1
	Gastrocnemius	530 (457–598) (4)	63.8 (47.3–78.8) (4)	4.1

* Taken from Crabtree & Newsholme (1972).

pathway is thought to be operative during anaerobiosis (Zammit & Newsholme, 1976). The ratios and the nucleoside diphosphokinase activities (for muscles of marine invertebrates that contain detectable activities of phosphoenolpyruvate carboxykinase) are presented in Table 2 and they indicate that such a negative correlation does exist.

For insect flight muscles, that of the waterbug is unusual, since the pyruvate kinase/phosphofructokinase activity ratio is considerably lower than for any other insect (Table 1). Furthermore, it is established that this muscle relies more on oxidation of fat than carbohydrate for energy production (Crabtree & Newsholme, 1972, 1975), so that a low activity of phosphofructokinase would be expected. However, the activity of fructose bisphosphatase is very high in these muscles and represents 25% of that of phosphofructokinase (see Newsholme & Crabtree, 1978). It is suggested that in this insect the fructose 6-phosphate–fructose bisphosphate substrate cycle is involved not in the regulation of glycolysis but in heat generation as has been shown for the bumble bee (Newsholme *et al.*, 1972; Clarke *et al.*, 1973). The waterbug flies at night when it might be necessary to generate heat to raise the thoracic temperature to approx. 30°C.

Effect of fructose bisphosphate on pyruvate kinase activities

It has been established that fructose bisphosphate activates pyruvate kinase from fish white skeletal muscle, bivalve adductor muscles (Somero & Hochachka, 1968; de Zwaan & Zandee, 1972) and turtle heart (Storey & Hochachka, 1973), but it does not affect the enzyme from the mantle muscle of the squid (Storey & Hochachka, 1976). In the present work, the effect of fructose bisphosphate on pyruvate kinase from muscles of a large number of animals has been investigated. The effect of fructose bisphosphate on pyruvate kinase was measured at concentrations of phosphoenolpyruvate that were below the K_m value for this substrate (i.e physiological concentrations; see Table 2). Furthermore, since very low concentrations of fructose bisphosphate can activate the enzyme from some animals (see Zammit & Newsholme, 1978), the extracts were always dialysed before assay and, in some cases (e.g. insect flight muscle, lizard muscles, pectoral muscles of domestic fowl, muscles of the mouse) pyruvate kinase was precipitated with $(NH_4)_2SO_4$ (the precipitate between 40 and 60%-satd. $(NH_4)_2SO_4$ was taken), the precipitate was taken up in extraction buffer and dialysed for 2–4h before the effect of exogenous fructose bisphosphate was tested. In some cases (e.g. muscles of the frog and lizard), activation of fructose bisphosphate was not observed unless low concentrations of phosphoenolpyruvate were used

Table 2. *Maximal activities of nucleoside diphosphokinase and the pyruvate kinase activity/2× phosphofructokinase activity ratios in muscles of marine invertebrates*

Activities of nucleoside diphosphokinase are taken from Zammit & Newsholme (1976) and the ratios are taken from Table 1. The animals are arranged in order of increasing activities of diphosphokinase. See Table 1 for systematic names. The correlation coefficients for the three equations $y = a+b \log x$, $y = a+bx$ and $y = a+b/x$ (where y is the ratio of activities and x is the diphosphokinase activity) are −0.78, −0.59 and 0.84 respectively.

Animal	Muscle	Nucleoside diphosphokinase activity (μmol/min per g at 25°C)	Pyruvate kinase activity/2× phosphofructokinase activity ratio
Sea cucumber	Pharyngeal	1.0	15.5
Sea anemone	Basilar	5.2	4.8
	Sphincter	5.4	4.2
Lobster	Claw	17.2	5.0
Horse-shoe crab	Leg	25.0	5.5
Squat lobster	Abdominal	27.4	6.6
Lobster	Abdominal	28.6	7.9
Horse mussel	Catch adductor	39.6	1.6
Top shell	Foot retractor	40.3	1.3
Oyster	Catch adductor	56.5	0.7
Pedunclulate barnacle	Shell closer	59.4	2.2
Horse mussel	Phasic adductor	70.1	1.0
Periwinkle	Foot retractor	91.3	2.5
Oyster	Phasic adductor	119.0	0.7
Sand gaper	Phasic adductor	159.2	1.3

and the extract had been either precipitated or dialysed. Fructose bisphosphate activated the enzyme from the muscles of all the marine invertebrates investigated, the heart, red and white muscle of the fish (dogfish, trout, goldfish and lungfish), and heart and skeletal muscles of the salamander, axolotl and lizard (see Table 3). Although a marked stimulation of pyruvate kinase activity from frog and toad heart was observed, there was little or no stimulation of the enzyme from the skeletal muscle of these animals (see Table 3). The reason for this difference between amphibian tissues is not known, but the lack of stimulation in skeletal muscle is not consistent with the hypothesis proposed below. Since precipitation and dialysis was not carried out in all cases and since the concentration of phosphoenolpyruvate used in the experiment was arbitrary (although it was always below the K_m value), caution must be exerted in the precise quantitative interpretation of the degree of stimulation by fructose bisphosphate. Activation was not observed with the enzyme from insect flight muscles and muscles (both skeletal and heart) of birds and mammals (Table 2). Lack of activation was observed in some of these muscles, despite the fact that the precipitation described above was used.

The effect of fructose bisphosphate is to decrease the apparent K_m of pyruvate kinase for phosphoenolpyruvate, in some cases by decreasing the sigmoidicity of the response of the enzyme to phosphoenolpyruvate (e.g. enzyme from axolotl heart and muscles of marine invertebrates; see Fig. 1 and Zammit & Newsholme, 1978). The effect of fructose bisphosphate was very specific: at concentrations of 0.5mM, the following compounds had no effect on pyruvate kinase activity, glucose 1-phosphate, glucose 6-phosphate, fructose 1-phosphate, fructose 6-phosphate, glyceraldehyde 3-phosphate, cyclic AMP and DL-glycerol 3-phosphate. However, glucose 1,6-bisphosphate (at a concentration of 0.5mM) activated the enzyme by no more than 10% of the effect of an equivalent fructose bisphosphate concentration.

The distribution of the activatory effect of fructose bisphosphate on pyruvate kinase from animals of different phyla is of considerable interest. The effect appears to be present in muscle of invertebrates, except for the insects (Table 2) and the squid (Storey & Hochachka, 1976). The mechanisms for supply of O_2 to muscles of insects and squids are highly developed [tracheal system in insects; closed high-pressure systemic circulatory system and efficient O_2 unloading by haemocyanin in cephalopods (Redfield & Goodkind, 1929)] and they are both considered to be very efficient. Thus it is suggested that the activatory effect of fructose bisphosphate on pyruvate kinase occurs in those invertebrate muscles in which the oxygen supply is poor. In such muscles, the change in the rate of glycolysis between rest and contractile activity should be large in order to provide sufficient energy from the inefficient process of anaerobic glycolysis. The activation of pyruvate kinase by fructose bisphosphate may provide a feed-forward control mechanism that ensures that the activity of

Table 3. *Effect of fructose bisphosphate on the activities and K_m values for phosphoenolpyruvate of pyruvate kinase from muscles of vertebrates amd invertebrates*
For the effect of fructose bisphosphate the concentrations of Mg^{2+}, K^+ and ADP were 7.5, 75 and 1 mM respectively, and the concentration of phosphoenolpyruvate was either 0.05 or 0.01 mM. In some experiments, the muscle extract was dialysed for 4–5 h with changes of buffer each hour, whereas in other experiments, indicated by an asterisk, the extract was precipitated with $(NH_4)_2SO_4$ and the precipitate taken up in buffer and dialysed as above. For the determination of the K_m values the concentrations of Mg^{2+} and K^+ were as above and the concentration of ADP was 2 mM, and in all cases K_m values were determined after $(NH_4)_2SO_4$ precipitation and dialysis (see the Materials and Methods section). In each case the results represent the mean of three separate experiments. All activities were measured at the pH optimum of each separate enzyme (e.g. properties of oyster pyruvate kinase were studied at pH 8.1).

		Pyruvate kinase activities (A_{340} unit/min per 5–10 μl of extract)		Ratios of activities, presence of fructose bisphosphate/absence of fructose bisphosphate	K_m of pyruvate kinase for phosphoenolpyruvate (mM)	
Animal	Muscle	No added fructose bisphosphate	0.2 mM-Fructose bisphosphate		No added fructose bisphosphate	0.2 mM-Fructose bisphosphate
Sea anemone	Basilar	—	—	—	0.1	0.07
Common oyster	Phasic adductor	0.021	1.12	53.3	0.40	0.05
Horse mussel	Phasic adductor	0.11	0.5	3.6	—	—
Lobster	Deep abdominal flexor	0.085	0.21	2.5	0.20	0.07
Horse-shoe crab	Leg	0.042	0.48	11.4*	0.06	0.03
Variegated scallop	Phasic adductor	0.032	0.050	1.6	0.05	0.03
Locust	Flight	0.030	0.032	1.1*		
	Hind-leg femoral	0.019	0.022	1.2*		
Rosechafer	Flight	0.025	0.020	0.8		
Dogfish	Red	0.018	0.058	3.2	—	—
	White	0.04	0.135	3.4	—	—
	Heart	0.025	0.048	2.0	—	—
Trout	Red	0.030	0.11	3.7	—	—
	White	0.080	0.17	2.1	0.07	0.03
	Heart	0.090	0.20	2.2	0.25	0.05
Goldfish	White	0.04	0.10	2.6	—	—
Lungfish	White	0.15	0.32	2.9	—	—
(*Protopterus*)	Heart	0.20	0.59	2.9	—	—
Frog	Gastrocnemius	0.023	0.029	1.3	—	—
	Heart	0.004	0.015	3.7	—	—
Toad	Gastrocnemius	0.048	0.053	1.1	—	—
(*Xenopus laevis*)	Heart	0.020	0.039	1.9	—	—
Axolotl	Skeletal	0.040	0.06	1.6	0.06	0.03
	Heart	0.025	0.2	8.0	0.43	0.05
Salamander	Skeletal	0.040	0.07	1.8	0.06	0.03
	Heart	0.011	0.022	2.0	0.09	0.06
Lizard	Skeletal	0.038	0.12	3.2*	—	—
	Heart	0.007	0.025	3.6*	0.11	0.05
Domestic fowl	Pectoral	0.14	0.14	1.0*	—	—
	Heart	0.010	0.012	1.2*	—	—
Mouse	Gastrocnemius	0.011	0.012	1.1	—	—
	Heart	0.009	0.010	1.1	—	—
Ox	Heart	0.125	0.125	1.0	—	—

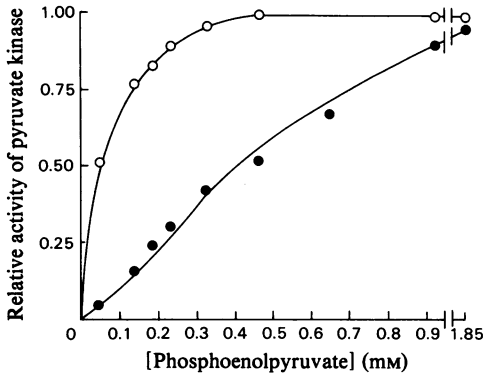

Fig. 1. *Plot of concentration of phosphoenolpyruvate against activity of pyruvate kinase for the heart muscle of the axolotl*
The extract was dialysed for 4h before assay, in the presence of 0.2mM-fructose bisphosphate (○) or in the absence of any added fructose bisphosphate (●).

pyruvate kinase is increased in relation to the change in activity of phosphofructokinase. The dependence of these muscles on glycolysis for energy generation may require a greater degree of integration between the various reactions than is necessary in the more aerobic muscles that are less dependent on anaerobic glycolysis for energy production. Furthermore, in muscles of marine invertebrates utilizing the 'succinate pathway', it may also be a mechanism for the initial rapid activation of pyruvate kinase at the start of a period of anaerobiosis. Such an initial activation would permit the rapid accumulation of alanine which is thought to be essential for the dual regulation of the activities of phosphoenolpyruvate carboxykinase and pyruvate kinase in these muscles (see Zammit & Newsholme, 1978).

For the vertebrates, the stimulation by fructose bisphosphate is observed in muscles of fish, amphibia (except for the skeletal muscles of the frog and toad) and reptiles. It is important to note that the effect is observed in the aquatic aerial breathing animals (e.g. lung-breathing fish, amphibians) as well as terrestrial lower vertebrates (e.g. salamander, lizard) (see Table 2). Thus the lack of stimulation by fructose bisphosphate does not appear to be related to the development of terrestrial life (or aerial breathing) either in the invertebrates or the vertebrates. The enzyme from muscles of birds and mammals is not activated by fructose bisphosphate. The reason for the loss of this property in the vertebrates may be similar to that in the invertebrates, since this loss coincides with the establishment of a complete double-circulatory system in the vertebrates. Such a system provides an efficient high-pressure perfusion of muscles, so that the O_2 supply to the aerobic muscles will be efficient both at rest and during exercise. Hence the loss of this property in these higher animals may be for similar reasons as in the insects and the squid (see above). However, it is established that some muscles in birds and mammals probably obtain most of their energy for contraction from anaerobic metabolism (e.g. pectoral muscle of the domestic fowl and pheasant, the adductor longus of the rabbit; see Crabtree & Newsholme, 1972, 1975), so that a high degree of integrated control of the glycolytic reactions is required. Despite this requirement, there is no activating effect of fructose bisphosphate on pyruvate kinase from these muscles. This suggests that another mechanism, which is predicted to be more effective than that of fructose bisphosphate activation, has been developed for the control of the activity of pyruvate kinase in concert with that of phosphofructokinase in the anaerobic muscles of the birds and mammals: the inhibition of both enzymes by phosphocreatine (Kemp, 1971, 1973) may provide the basis for such a concerted mechanism of control.

Recently, it has been shown that, whereas pyruvate kinases from skeletal muscle of the adult rat and chicken exhibit no response to fructose bisphosphate, the enzymes from the foetal rat and chicken muscle are stimulated by this compound (Guguen-Guillouzo *et al.*, 1977; Harris *et al.*, 1977). A marked activation by fructose bisphosphate was observed on the 15th day of gestation in the rat, but this was almost non-existent by the 21st day. The difference in response appears to be due to a change in isoenzyme pattern during development. This suggests that, although the genetic capability for the synthesis of a fructose bisphosphate-sensitive pyruvate kinase is present in birds and mammals, the postulated alternative control mechanism (see above) is preferred for the muscles in the adult animal. Phillips & Ainsworth (1977) have shown that rabbit muscle pyruvate kinase can be activated by fructose bisphosphate, but only at unphysiologically high concentrations (>1mM). This finding is consistent with the view that the enzyme in adult birds and mammals has not lost its binding site for fructose bisphosphate but only the sensitivity of this site for binding of the activator.

We thank Professor R. R. Porter, F.R.S., for his interest and encouragement, and Professor E. J. Denton, F.R.S., for use of facilities at the Laboratory of the Marine Biological Association, Plymouth. This work was supported by the Science Research Council (B/RG/72217).

References

Awapara, J. & Simpson, J. W. (1967) *Annu. Rev. Physiol.* **29**, 87–112

Beis, I. D. & Newsholme, E. A. (1975) *Biochem. J.* **152**, 23–32

Bücher, Th. & Pfleiderer, G. (1955) *Methods Enzymol.* **1**, 435–440

Clark, D. G., Bloxham, D. P., Holland, P. C. & Lardy, H. A. (1973) *Biochem. J.* **134**, 589–597

Crabtree, B. (1976) *Biochem. Soc. Trans.* **4**, 1046–1048

Crabtree, B. & Newsholme, E. A. (1972) *Biochem. J.* **130**, 697–705

Crabtree, B. & Newsholme, E. A. (1975) in *Insect Muscle* (Usherwood, P. N. R., ed.), pp. 405–500, Academic Press, London

Crabtree, B., Higgins, S. J. & Newsholme, E. A. (1972) *Biochem. J.* **130**, 391–396

de Zwaan, A. & Zandee, D. I. (1972) Comp. Biochem. *Physiol. B* **43**, 47–54

Guguen-Guillouzo, C., Szajnert, M. F., Matie, J., Delain, D. & Schapira, F. (1977) *Biochimie* **59**, 65–71

Harris, W., Days, R., Johnson, C., Finkelstein, I., Stallworth, J. & Hubert, C. (1977) *Biochem. Biophys. Res. Commun.* **75**, 1117–1121

Kemp, R. G. (1971) *J. Biol. Chem.* **246**, 245–252

Kemp, R. G. (1973) *J. Biol. Chem.* **248**, 3963–3967

Mustafa, T. & Hochachka, P. W. (1971) *J. Biol. Chem.* **246**, 3196–3203

Newsholme, E. A. & Crabtree, B. (1978) *FEBS Symp.* **42**, 285–295

Newsholme, E. A. & Taylor, K. (1969) *Biochem. J.* **112**, 465–474

Newsholme, E. A., Crabtree, B., Higgins, S. J., Thornton, S. D. & Start, C. (1972) *Biochem. J.* **128**, 89–97

Opie, L. H. & Newsholme, E. A. (1967) *Biochem. J.* **103**, 391–399

Phillips, F. C. & Ainsworth, S. (1977) *Int. J. Biochem.* **8**, 729–735

Redfield, A. C. & Goodkind, R. (1929) *J. Exp. Biol.* **6**, 340–349

Saz, H. J. (1971) *Am. Zool.* **11**, 125–135

Somero, G. N. & Hochachka, P. W. (1968) *Biochem. J.* **110**, 395–400

Storey, K. & Hochachka, P. W. (1973) *J. Biol. Chem.* **249**, 1423–1429

Storey, K. & Hochachka, P. W. (1976) *Comp. Biochem. Physiol. B* **52**, 187–191

Zammit, V. A. & Newsholme, E. A. (1976) *Biochem. J.* **160**, 447–462

Zammit, V. A. & Newsholme, E. A. (1978) *Biochem. J.* **174**, 979–987